**AN ABSTRACT OF THE THESIS OF**

Mueez Ahmad for the degree of Master of Science in Veterinary Medicine presented on May 2, 1990.

Title: Application Of Monoclonal Antibody For The Immunohistochemical Detection Of Newcastle Disease Virus In The Chicken

Redacted for Privacy

Abstract approved: Masakazu Matsumoto

The detection and identification of Newcastle disease virus (NDV) has never been straightforward. Serologically indistinguishable pathotypes produce a wide spectrum of disease that ranges from a severe respiratory, neurologic, or enteric disease with high mortality to an asymptomatic infection. Extensive use of ND vaccine strains further complicates the serological differentiation of field isolates. Recently, monoclonal antibodies (MAbs) have been produced in some laboratories which are capable of detecting antigenically different pathotypes (Russell 1983). Our major objective of this study was to apply immunohistological techniques for rapid detection of NDV antigen using monoclonal or polyclonal antibody in infected chickens. Two methods of immunocytochemistry, immunofluorescence (IF) and alkaline phosphatase-monoclonal anti-alkaline phosphatase (APAAP), were used to demonstrate

Newcastle disease virus (NDV) antigen in impression smears and formalin-fixed, paraffin-processed sections of lungs and tracheas obtained from infected chickens. Specific immunofluorescent and alkaline phosphatase staining with a monoclonal antibody was detected within the cytoplasm of epithelial cells of trachea or secondry and tertiary bronchi. The results were compared between the above mentioned techniques and virus isolation.

Immunofluorescent staining of impression smears was the easiest approach. Specific staining pattern with MAb was confirmatory in comparison with polyclonal antibodies which generally showed varying degree of nonspecific staining. The staining with APAAP was permanent, and it was possible to correlate histopathological changes with actual location of antigen in situ. These techniques appeared potentially useful for rapid and definitive diagnosis of Newcastle disease in the future.

# APPLICATION OF MONOCLONAL ANTIBODY FOR THE IMMUNOHISTOCHEMICAL DETECTION OF NEWCASTLE DISEASE VIRUS IN THE CHICKEN

by

Mueez Ahmad

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for
the degree of

Master of Science

Completed May 2, 1990

Commencement June 1990

APPROVED:

Redacted for Privacy

Associate Professor of Veterinary Medicine in charge of major

Redacted for Privacy

Dean, College of Veterinary Medicine

Redacted for Privacy

Dean of Graduate School

Date thesis is presented May 2, 1990

**ACKNOWLEDGEMENT**

I want to express my sincere gratitude to my committee members, and to the many individuals who aided me in my research efforts, and thus added to the quality of this thesis. I specifically would like to acknowledge my major advisor, Dr. Masakazu Matsumoto who provided research guidance and a professional review of my manuscript. I would also like to thank Dr. Wallner Eva Pendelton for giving me professional guideline in the field of avian pathology.

My gratitude also goes to the many people who gave me research leads, articals, or books, or materials related to my research, including: Dr. M. Burgh, of Southeast Poultry Research Laboratory USDA, Athens Georgia and Dr. Senne, the National Veterinary Service Laboratory, U.S.D.A., Ames, Iowa. I also wish to acknowledge, and offer my appreciation to the USAID-GOP to provide funding for my research. I would like to express love and appreciation to my wife Darlene Rene Mueez.

**TABLE OF CONTENTS**

## LIST OF FIGURES

## LIST OF TABLES

# APPLICATION OF MONOCLONAL ANTIBODY FOR THE IMMUNOHISTOCHEMICAL DETECTION OF NEWCASTLE DISEASE VIRUS IN THE CHICKEN

## Chapter I

## LITERATURE REVIEW

## NEWCASTLE DISEASE

**History** Newcastle disease virus (NDV) is the causative agent of a major disease of poultry. The first report of a disease amongst chickens which, according to the clinical signs, retrospectively appears to have been Newcastle disease (ND) stems from an island of Java in 1926 (Kraneveld, 1926), but the disease was first recognized as a filtrable virus infection after an outbreak at Newcastle-upon-Tyne England (Doyl, 1927). Similar outbreaks were reported in Korea, India, and the Philippines within a few years. Initially recognized ND was in different parts of world as a highly pathogenic disease with a level of mortality up to 98-100%. These early outbreaks were characterized by a disease with the incubation period from 4-11 days with an acute and frequently lethal infection of chickens. The respiratory and nervous signs were apparent. Hemorrhagic lesions of the digestive tract were also observed.

**Virus Classification** The virus has been classified in the family Paramyxoviridae which includes three genera Morbillivirus, Pneumovirus and Paramyxovirus. The genus paramyxovirus includes the mammalian parainfluenza viruses, mumps virus and avian paramyxoviruses (PMVs) (Alexander & Collins 1981). The avian paramyxoviruses have been mainly

grouped serologically by their similarities in hemagglutination inhibition (HI) tests (Alexander, 1980). These viruses have been loosely classified (into 9 serotypes PMV-1 to PMV-9) on the basis of hemagglutination inhibition (HI) and virus neutralization index (NI) properties of type specific antisera (Alexander, 1982; Alexander et al., 1983). The established serotypes are quite distinct, but some cross reactions between serotypes have been shown by HI tests between PMV-1 and PMV-4 (Kessler, et al., 1979), PMV-1 and PMV-8, PMV-1 and PMV-9, PMV-3 and PMV-8, PMV-3 and PMV-9, PMV-4 and PMV-8 (Alexander, 1986). All the viruses responsible for Newcastle disease in poultry and other birds are grouped in PMV-1 (Alexander, 1982).

**The Virus** Newcastle disease virus (NDV) is an enveloped RNA virus with a non-segmented, single-stranded genome of negative polarity surrounded by protein to form helical nucleocapsid which in turn is enclosed within an envelope of modified cell membrane of host origin (Kingsbury, & Darlington, 1968). The virus particles are pleomorphic and roughly spherical having considerable variation in size ranges from 100-200 nm in diameter. Some particles may be as large as 600 nm when viewed by an electron microscope with negative contrast (Bang, 1948; Rott, et al., 1961). The virion can be viewed as consisting of two structural units, the ribonucleoprotein or

nucleocapsid, and the envelope with its surface projections. The nucleocapsid is a single left-handed helical structure consisting of RNA and nucleocapsid protein (NP) (Finch, 1970). Two other proteins, the phosphoprotein (P) and the large protein are also associated with nucleocapsid and recognized only by immune electron microscopy (Portner, & Murti, 1986). The envelope consists of a lipid bilayer with spike-like surface projections of hemagglutinin-neuraminidase (HN) and fusion proteins. The lipid bilayer is modified host cell plasma membrane having HN viral proteins (Klenk, & Choppin, 1970). The neuraminic acid-containing glycolipid are removed by the viral neuraminidase (Klenk, et al., 1970).

**Viral Proteins** The NDV genome encodes for the six gene products listed in order from 3' end of minus strand: a nucleocapsid protein; a phosphoprotein; a membrane or matrix protein; a fusion protein ($F_0$ uncleaved, $F_1$,$F_2$ cleaved); hemagglutinin-neuraminidase; and a large protein (Chambers, et al., 1986).

Nucleocapsid protein The NP is a major structural component of the nucleocapsid, making the helical nucleocapsid structure.

Phosphoprotein Associated with nucleocapsid, phosphorylation of the protein plays some regulatory role in transcription and replication (Colonno, & Stone, 1976).

Membrane or Matrix protein The M protein has a crucial role in virus assembly, interacting with both nucleocapsid and viral membrane plasma membrane in which the F and HN glycoprotein are anchored (Nagai, et al., 1976; Matsumoto, 1982).

Fusion protein This protein is involved in the fusion between virus envelope and the cell membrane. A post translational cleavage of inactive precursor, $F_o$, into two disulfide-bonded polypeptide, $F_1$ and $F_2$ , is necessary to produce fusion-active F protein and thereby yield infectious virion (Nagai, et al., 1976; Scheid, & Choppin, 1977).

Hemagglutinin-Neuraminidase NDV has both hemagglutinin (HA) and neuraminidase (NA) activities residing on a single glycoprotein spike of the envelope (Schied, & Choppin, 1973). Hemagglutinin provides the attachment functions to neuraminic acid (sialic acid) containing glycoprotein receptors on erythrocytes or target cells. The neuraminidase is involved in elution of the virus from the cell surface (Scheid, & Choppin, 1973).

Large protein The L protein of NDV, together with P protein is essential for viral RNA synthesis (Hamaguchi, et al., 1983).

**Virus replication** The first step in virus infection is attachment of hemagglutinin component of NDV to the

neuraminic acid receptor on the target cell in the presence of salt (Levine, & Sagik, 1956). The penetration of the viral genomic material (nucleocapsid) into the cytoplasm is mediated by the fusion of the viral envelope with the lipid bilayer of the target cell (Meiseiman, et al., 1967). The fusion protein is involved in this process (Nagai, et al., 1976; Scheid, & Choppin, 1977). The NDV nucleocapsid carries RNA-dependent RNA polymerase into the cytoplasm of the target cell, which initiates primary transcription. The primary transcription yields at least six mRNA, which are capped at 5' ends and polyadenylated at their 3' ends (Collins, et al., 1980). These mRNAs are subsequently translated into viral proteins (Kingsbury, 1970). The site of RNA replication is the cytoplasm (Choppin, & Compans, 1975). The genome replicates first by producing a full length (+) RNA. The second half of replication involves making a full-length genomic (-) RNA from the full length (+) RNA (Kurilla, et al., 1985; Banerjee, 1987). The virus assembles its components at the plasma membrane of infected cells in association of M protein and produces infectious virus by budding (Feller, et al., 1969; Nagai, et al., 1975). Biologically active proteins are first detected 3-4 hours after infection by use of fluorescent antibodies (Rott, 1964). The specific antigen accumulates and structures identical in appearance to the mature virion appear just within the cell membrane 4 hour post-inoculation

(Wheelock, 1963). Liberation of virus starts 4 hr postinfection and can continue up to 8 hr without destruction of the vital processes of the infected cell (Wheelock, 1963).

**Pathogenesis** The pathogenesis of NDV depends on a number of factors, including the species of bird, age, immune status, route of exposure and dose of infecting virus. The most important are the virulence and tropism of the viruses. NDV strains are broadly classified into Lentogenic (e.g. B1, F, or LaSota strain), Mesogenic (e.g. Roakin strain), and Velogenic forms (e.g. GB, Herts, Milano strain) based on the virulence expressed by mean death time of embryonated eggs, intracerebral pathogenicity in one-day-old chickens, intravenous pathogenicity index in six-week-old chickens and plaque formation on chick embryo fibroblast monolayers (Allan, et al., 1973). NDV has also been characterized into four pathotypes based on the clinical signs in chickens: 1) Viscerotropic velogenic (VVND), Doyle's form or Asiatic NDV; 2) Neurotropic velogenic (NV), Beaches form; 3) Mesogenic, Beaudette form; and 4) Lentogenic, Hitchner's form. In addition, a fifth pathotype, the avirulent alimentary tract associated virus (the Ulster type) was also added (Beard & Hanson, 1984). It has been demonstrated that monoclonal antibodies are a

powerful tool for quick and accurate classification of NDV isolates into groups sharing epizootiological and biological properties (Russel, & Alexander, 1983).

**Sequence of organs involved** A sequence of events following introduction of NDV into the chicken is initiated by multiplication of the virus at the site of introduction (Asdel, & Hanson, 1960). This is usually the conjunctiva or the respiratory or alimentary tracts (Kato, 1977). A second cycle of multiplication occurs in visceral organs following the liberation of the virus into the blood stream. Pathological events depend on the type of NDV strain involved. Lentogenic strain infection is not lethal, however histopathological changes occur in the chicken trachea via the aerosol, intratracheal, or air sac route of inoculation. There is deciliation of epithelial cell, congestion and inflammatory cell infiltration in the tracheal mucosa at day 2 post inoculation. Severity of these signs is increased by day 4 and there are numerous vacuolated epithelial cells in the mucosa. Regeneration of epithelium is usually observed after a week (Kotani, et al., 1987). After the intranasal inoculation of the mesogenic strain, the destruction of mucus cells in individual acini in the middle turbinate are observed. The virus rapidly spreads to the neighboring ciliated and goblet cells and acini. There is destruction of the mucosa, infiltration of

inflammatory cells and loss of cartilage basophilia by the second day post inoculation. The inflammatory reaction subsides by day 5 and by day 8 the epithelium is normal. There is liberation of virus into the blood stream, followed by multiplication of virus in visceral organs (Bang, et al., 1974). Thus, by 44 hours post infection high virus titer is found in the kidney, spleen, lung and bursa. In addition, a substantial amount of virus is present in the duodenum, trachea, pancreas and brain (Singh, & El-Zein, 1978). The histological changes include hyperemia, edema, hemorrhage and other vascular changes which consist of hydropic degeneration of media, hyalinization of capillaries and arterioles, development of hyaline thromboses in small vessels and finally necrosis of endothelial cells of vessels (Beard, & Hanson, 1984).

Gross lesions There is no single gross lesion which is pathognomonic for Newcastle disease. The lesions of influenza virus, infectious laryngotracheitis and infectious bronchitis may resemble the lesion seen in some form of ND. However, history of sudden onset and symptoms within the flock can be highly indicative of infection with NDV of velogenic or the mesogenic type (Orsborn, 1977). A diagnosis based on clinical evidence should always be supported by laboratory studies.

**Diagnosis** The detection and identification of Newcastle disease virus (NDV) has never been straightforward. Serologically indistinguishable pathotypes produce a wide spectrum of disease that ranges from a severe respiratory, neurologic, or enteric disease with high mortality to an asymptomatic infection. Extensive use of ND vaccine strains further complicates the serological differentiation of field isolates. There are a number of procedures employed for NDV diagnosis.

**Virus Isolation and Identification** The virus can be isolated from a variety of tissues e.g. lung, trachea, brain, spleen, liver, kidney and bone marrow. For routine isolation purpose tissue samples or swabs are obtained from the trachea and cloaca (Lancaster, 1966). The virus can be readily propagated in tissue culture of avian origin or in specific-pathogen-free embryonated eggs (Beard, & Hanson, 1984). Isolation of NDV may be confirmed by serological methods.

The isolates of ND may be differentiable on the basis of their virulence (Kaleta, et al., 1979), physical characteristic, and difference in antigenicity (Russell, & Alexander, 1983). Currently accepted virus characterization involves an assessment of virulence by one of the following in vivo tests (Hanson, 1980).

1) Mean death time of embryo.

2) The intracerebral pathogenicity index.

3) The intravenous pathogenicity index.

**Serology** Several serological procedures are currently being used to demonstrate virus in culture and antibody in paired sera (Hanson, 1980; Alexander, 1988).

1) Hemagglutination.

2) Hemagglutination inhibition.

3) Virus neutralization in embryonating eggs.

4) Plaque neutralization.

5) Immunodiffusion.

6) Complement fixation.

7) Enzyme-linked immunoabsorbent assay.

8) Fluorescent antibody test.

9) Electron microscopy.

**Monoclonal Antibodies** Monoclonal antibodies (MAbs) bind to a single antigenic determinant or epitope of the target antigen. When prepared against viruses or virus antigens, MAbs may be deployed to detect antigenic variation in viruses which appeared identical by the use of polyclonal antisera (Russell, & Alexander, 1983). By 1980 several laboratories produced MAbs for such virological studies of Newcastle disease virus as: 1) Epidemiological studies; 2) Antigenic variation; 3) Virulence marker; 4) Pathotyping; 5) Study of virus infected cell; 6) Immuno diagnosis (Meulemans, et al., 1987; Oxford, 1982).

Russell and Alexander (1983) produced a panel of MAbs to lentogenic Ulster 2C. Nine MAbs placed 40 strains of PMV-1 into eight antigenically distinct groups that shared biological and epizootiological properties, but these MAbs did not entirely delineate the strains into distinct pathotypes. A panel of MAbs was also used to differentiate NDV from avian paramyxovirus infecting racing pigeons in Europe (Alexander, et al., 1984). Iorio et al., (1983) found that at least four determinants were delineated on HN, two of them overlapping. It was observed that regions on HN and F contain antigenically conserved and variable epitopes that explain both the monotypical nature of NDV by conventional serology and vaccinal cross protection as well as antigenically distinct grouping (Iorio, & Bratt, 1983; Iorio, et al., 1986). Meulemans, et al., (1987) produced monoclonal antibody against NDV glycoprotein and used them as strain markers to differentiate NDV strains.

MAbs were also produced to the internal proteins of NDV. Using competitive binding assays, two non-overlapping antigenic sites were found on the membrane protein, four on the nucleocapsid associated protein, and two on nucleocapsid protein. Reactivity to heterologous NDV strains indicated both highly conserved and variable antigenic sites on M and P proteins. NP appeared more antigenically stable (Nishikawa, et al., 1987).

Many research laboratories have been working to develop

MAbs to a unique epitope expressed exclusively on either virulent or avirulent strains. These MAbs would be useful for pathotyping of NDV isolates. Srinivasappa, et al. (1987) developed murine MAb designated AVS-1 that showed high titer in HI test with only lentogenic vaccine strain. Erdei, et al., (1987) produced LaSota strain specific MAb named La-1. On the bases of ELISA reactivity, out of 300 lentogenic, mesogenic and velogenic strains La-1 bound only with LaSota strain. Lana, et al., (1988) successfully generated a battery of MAbs for differentiation of NDV strains and pathotyping.

**Diagnostic Immunohistochemistry** In recent years immunohistochemical techniques have gained popularity . The prospect of a rapid facile means of laboratory diagnosis with sensitivity and specificity that would make it superior to all existing technologies was seen as dawning of a new era in diagnostic pathology and research. There are two major branches of immunochemistry, enzyme immunochemistry and immunofluorescent histochemistry. In general, antibodies can be either chemically or immunologically conjugated with enzyme or fluorescent markers. The specificity of antibody is then exploited to locate antigens in histologically prepared tissue sections. For routine laboratory use, a single detection system for all antigens and tissue would be a choice. The indirect and multilayer

peroxidase antiperoxidase (PAP), Alkaline phosphatase antialkaline phosphatase (APAAP), and avidin biotin complex (ABC) are examples of most widely used techniques.

**Alkaline Phosphatase Immunocytochemistry** The potential value of alkaline phosphatase as an antibody label was investigated by Avrameas (1969). Subsequent studies have shown that this antigen detection system is efficiently applicable to the routinely processed tissue with adequate preservation of morphological characteristic (Sternberger, 1986; Mason & Sammons, 1978). In addition the tissues having high endogenous peroxidase activity could be stained with this alternative enzyme system (Cordell, et al., 1984). The basic principle applied in this method is that alkaline phosphatase is bound to the primary monoclonal antibody via an unlabeled bridging antibody followed by using immune complex of alkaline phosphatase and monoclonal anti-alkalinephosphatase (APAAP complex) (Fig. 1). A variety of substrate may be utilized to reveal the enzyme reaction in the APAAP procedure e.g Fast Red. In our procedure, the enzymic hydrolysis of phosphate ester of 6-bromo-2-hydroxy-3-naphthoic acid (buffer substrate solution) in the presence of hexazotized triaminotrimethyl-tripheylmethane produced a vivid scarlet coloration at the site of enzyme activity (Mason 1985).

**Immunofluorescent Technique** The indirect fluorescent antibody technique was originally described in 1954 (Coons, et al., 1954). The primary antibody is an unlabelled polyclonal or monoclonal directed against the antigens present in the fixed cells. The secondary antibody is usually an FITC-labelled polyclonal antibody directed against the gamma globulin fraction of the serum from the animal species in which the primary antibody was raised. This technique is simple and involve two steps. The first step is the binding of primary antibody to the antigen. Following incubation and washing, the slide is dried carefully. The second step involves the application of FITC conjugated secondary antibody on tissue. After optimal incubation, washing, drying and mounting with buffered glycerol, the slide is examined through a fluorescent microscope at magnification of 200x to 400x.

**Immunoenzymatic (APAAP) versus Immunofluorescent (IFT)** Both techniques are very similar in term of sensitivity (Mason, et al., 1986). The indirect immunofluorescent technique is simple and relatively rapid to perform. However fluorescent labels fade on excitation and storage. There is more chances of nonspecific staining. The IFT requires fluorescent microscopy. The APAAP technique produces permanent staining and can be visualized simultaneously with a counter stain under a standard

microscope. This technique allows the study of histopathological changes in the tissue.

Chapter II

# APPLICATION OF MONOCLONAL ANTIBODY FOR THE IMMUNOHISTOCHEMICAL DETECTION OF NEWCASTLE DISEASE VIRUS IN THE CHICKEN

## SUMMARY

A comparative study was conducted to evaluate the sensitivity and specificity in detecting NDV antigen in infected tissues between the virus isolation (VI) and immunocytochemistry. The comparison was also made between the use of monoclonal (MAb) versus polyclonal antibody (PAb). Two methods of immunocytochemistry, immunofluorescence (IF) and alkaline phosphatase monoclonal anti-alkaline phosphatase (APAAP), were used to demonstrate Newcastle disease virus (NDV) antigen in impression smears or formalin-fixed, paraffin-processed sections of lungs or tracheas obtained from infected chickens.

Specific immunofluorescence and alkaline phosphatase staining was detected with the monoclonal antibody frequently within cytoplasm of epithelial cells of the trachea and less frequently in tertiary bronchi and air capillaries. Virus isolation of most samples was positive from 8 hr post inoculation up to 120 hr. Specific immunohistostaining was positive in all samples that were VI positive.

Immunofluorescent staining of impression smears was the simplest approach. Specific staining pattern with MAb was confirmatory in comparison with PAb which generally showed varying degrees of nonspecific staining. The staining with APAAP was permanent, and it was possible to correlate

histopathological changes with actual localization of antigen *in situ.* These techniques appeared potentially useful for rapid and definitive diagnosis of Newcastle disease.

## INTRODUCTION

Newcastle disease is a highly contagious and destructive malady of avian species including chickens, turkeys, and pigeons (Hanson, 1984). The etiological agent Newcastle disease virus (NDV) has long been known as one of the most diverse and deadly avian pathogens belonging to the avian paramyxoviruses group of enveloped negative-stranded RNA viruses (Alexander, 1980). These avian paramyxoviruses have been loosely classified into 9 serotypes PMV-1 to PMV9 based on the hemagglutination inhibition and virus neutralization properties of type specific antisera (Alexander, 1982; Alexander, et al., 1983). NDV is grouped into PMV-1 (Hanson, 1978). The broad host spectrum, world wide distribution and severe losses in the poultry industry make the NDV infection an important issue (Lancaster, & Alexander, 1975). A wide range of strains have been isolated which differ markedly in pathogenicity (Russell, & Alexander, 1983). In the chicken the disease takes four distinguishable forms (Hanson, 1978; Alexander, & Allan, 1974). Doyle`s form (Doyle, 1927). This form is characterized by sudden mortality, usually 90% or more and birds are often found dead without showing any clinical signs. Sometimes oedema of the eyes, diarrhea and torticollis are found. On postmortem examination, marked hemorrhagic lesions may be seen in the intestine. This type

of disease has been termed viscerotropic velogenic Newcastle disease (VVND) (Hanson, et al, 1973; Spalatin, et al, 1976). Beach`s form (Beach, 1944). In this form of the disease, infected chickens show respiratory distress followed by nervous signs. Mortality is usually 10-15%, no diarrhea or intestinal lesions commonly observed. Viruses causing this form of disease have also been termed neurotropic velogenic NDV. Beaudette`s form (Beaudette, & Black, 1946). This is a respiratory form but occasionally nervous signs are observed. Mortality is usually low in adult birds. This form is caused by mesogenic strains of NDV. Hitchner`s form (Hitchner & Johnson, 1948). A very mild or inapparent disease caused by lentogenic strains of NDV. Besides these four distinct forms, mixed type of infection may also occur.

The effective disease control programs minimize the losses, but ND is still a big threat to our poultry industry in various forms. Rapid detection and differentiation of virulent and avirulent strains in the chicken is imperative in order to initiate appropriate control measures. In case of subclinical infection, early diagnosis of ND could be helpful in disease prevention programs as well as proper rescheduling of vaccination. Mouse monoclonal antibodies against NDV which have been developed in some research laboratories have considerable diagnostic importance. These MAbs are capable of detecting antigenically different pathotypes of NDV (Lana, et al.,1987; Srinivasappa, et al.,

1986). Currently immunohistochemical techniques with monoclonal antibodies has made possible a new era of avian diagnosis. Successful diagnostic procedures have been established for infectious bursal disease and avian adeno virus (Cho, et al., 1986; Sadasiv, et al.,1989; Cavanagh, 1985). The basic purpose of this study was to develop a rapid diagnostic method for ND by localizing NDV in infected tissue of chickens with immunohistochemical techniques.

## MATERIAL AND METHODS

Fertile eggs and chicken Specific-pathogen-free (SPF) fertile eggs, obtained from either, SPAFAS, Inc. (Norwich, Connecticut), or HY-VAC Laboratory Egg Co. (Gowrie, Iowa), were used throughout the experiment. Chicken hatched from SPF eggs were reared in isolation facilities.

Virus LaSota vaccine strain of NDV, Clone Vac-30 was obtained from Intervet America Inc. (Millsboro, Delaware), and propagated in SPF chicken embryos. The harvested chorioallantoic fluid (CAF) was dispensed in Hank's balanced salt solution (HBSS), (Gibco Co., Grand Island, New York), and kept at -70 C°.

Infection of chicks One-week-old SPF chicks were tagged with aluminum clips, randomized and separated in two different room for control and infected. The infected chicks were distributed into the groups of six chicks each in separate cages. The virus dilutions were made in HBSS and .1 ml of each dilution was inoculated intratracheally with the help of 1 ml tuberculin syringe having 18 gauge blunt needle.

Virus isolation Lungs and tracheas were finely minced with sterile scissors and approximately 20% (w/v) suspension made in brain heart infusion broth (Difco, Detroit, Michigon), containing penicillin (250 IU/ml) and streptomycin (250 μg/ml). Hemagglutination activity of NDV

was demonstrated in bacteria-free allantoic fluid by HA test.

Antiserum Polyclonal antiserum produced in the chickens inoculated with LaSota strain was obtained from Dr. M. Burgh, of Southeast Poultry Research Laboratory USDA, Athens Georgia. Monoclonal antibody (MAb) against paramyxovirus, serotype 1, designated as B79, has been described, (Lana and Snyder, 1988) and was obtained as mouse ascitic from Dr. Senne, the National Veterinary Service Laboratory, U.S.D.A., Ames, Iowa.

Tissue Preparation Impression smears were prepared as follows; The freshly cut tissue surface was pressed against a clean glass slide, air dried and fixed in chilled absolute acetone for 10 minutes. Samples for paraffin embedding were placed in 10% neutral buffered formalin overnight at room temperature and processed according to the standard procedure (Sumner, 1988). Paraffin sections of lungs or tracheas obtained from control or infected groups were cut, deparaffinized in two changes of xylene and rehydrated by passing through graded alcohol as described (Sumner, 1988).

Immunohistochemistry For immunofluorescent studies, acetone-fixed impression smears were incubated with primary antibody (1:80 dilution of MAb or 1:100 dilution of PAb) for 1 hr, rinsed with PBS and a 1:20 dilution of fluorescein-labeled affinity- purified antibodies against mouse or chicken IgG (Kirkegaaed & Perry Lab. Inc., Gaithersburg,

Maryland) was applied. For immunoenzyme staining, processed paraffin sections were covered with 20% normal rabbit serum for 1 hr and drained. MAb in 1:100 dilution was applied to the sections, incubated for 1 hr at 37 C and rinsed with PBS. The sections were further stained with alkaline phosphatase-monoclonal anti-alkaline phosphatase (APAAP) immunostaining kit (Zymed Laboratories, Inc., South San Francisco, Calif.) according to the procedure recommended by the manufacturer (Fig. 1). The optimum dilutions of both primary and secondary antibodies were achieved by the checker board type of titration.

Experiment 1 The stock virus was diluted ten fold, and each dilution was inoculated 0.1 ml intratrachealy in six groups, each having six one-week-old SPF chicks. An uninoculated group was kept in a separate room. Clinical signs were recorded daily up to five days to determine $ID^{50}$. Tissue samples were collected at 5 days post inoculation for immunochemistry and virus isolation.

Experiment 2 One-week-old SPF chicks were intratracheally inoculated with 1000 $ID^{50}$ of stock virus per chicken. An uninoculated control group was placed in separate isolation facility. Tissue samples of lungs and tracheas were collected from control and infected chicks at various time intervals to be processed for paraffin embedding, impression smears and virus isolation. Impression smears were stained with IF using MAb or PAb.

Paraffin sections were stained with APAAP using MAb.

Experiment 3 One-week-old SPF chicks were infected with 1000 $ID_{50}$ of stock virus per chicken by the intratracheal route. Tissue samples of lungs and tracheas were collected from control and infected chicks at various time intervals up to 7-days. These samples were processed for paraffin embedding and impression smears as described above.

## RESULTS

Experiment 1 The results of this experiment are summarized in Table 1. The virus was isolated from lungs and tracheas of all groups. The uninoculated control group showed neither embryo mortality nor HA activity. Immunofluorescent staining of lung and trachea smears obtained from most infected groups were positive. The number of stained epithelial cells was much higher in tracheas than in lungs. Immunofluorescence staining with Polyclonal antibody showed higher intensity in positive cells than MAb did; however, the degree of nonspecific fluorescence was higher with PAb (Fig. 3). A sharp granular cytoplasmic fluorescence pattern was indicative of positive results (Fig. 3, 4, 5). With MAb, background staining was negligible but intensity of fluorescence was less and quenching was obvious with some samples (Fig. 6, 7). The fluorescence pattern was different from that with PAb; a diffuse and speckled cytoplasmic fluorescence was commonly observed with positive samples (Fig. 6). PAb showed higher intensity as compare to MAb (Fig. 2).

Experiment 2 The results of this experiment are summarized in Table 2. Most of the paraffin sections of the lung and trachea from 8hr PI to 120 hr PI were APAAP-positive with APAAP staining (Fig. 9 to 20). APAAP staining with MAb was detected in the cytoplasm of epithelial cells

of trachea (Fig. 9), secondary or tertiary bronchi (Fig. 19) and the air capillaries (Fig. 20). In general the tracheal epithelium was more frequently stained as compared to the parabronchi (tertiary bronchi) or the air capillaries. Immunofluorescent staining of lung and tracheal smears was detected in epithelial cells. However tracheal smears had higher numbers of stained cells than lung. As seen in the previous experiment, there was a difference in the fluorescence pattern between PAb and MAb (Fig. 1, 3, 6).

Virus isolation from most of samples was positive from 8 hr post inoculation (PI) up to 120 hr (Table 2). Specific immunohistostaining was positive in all samples that were VI positive.

Experiment 3 The observation of APAAP staining of paraffin processed lungs and tracheas and immunofluorescence staining of impression smears were similar as in previous experiment. Since we did not find any positive sample at 10 day PI in the previous experiment, the tissue samples were taken only up to seven days. The results were summarized in table 3.

4 hour PI: No histologic evidence of infection observed, however one out of five infected chicken tracheal sample was positive for VI, IF and APAAP tests (Table 2).
8 Hour PI: The initial site of virus localization was the cytoplasm of the tracheal epithelium. Most of the samples taken at 8 hr PI exhibit partial deciliation along with the

localization of antigen in cytoplasm as a red colored staining (Fig 9). 24 hour PI: There was complete deciliation of the tracheal epithelium. Separation of mucosa at some parts was observed along with intra and extra cellular localization of virus as red staining with APAAP (Fig. 10). 48 hour PI: Disappearance of mucus from acinar cells or goblet cells. The goblet cell vacuolation was observed commonly (Fig. 11). There was marked edema and lymphocytic infiltration and congestion of blood vessel at the site of infection (Fig. 12, 13). 120 hour PI: Histopathological changes reached peak at 120 hr PI. There was hyperplasia, necrosis and sloughing of epithelium (Fig. 15,16, 17). 10 day PI: The intensity of the lesions subsided by 7 day PI (Fig. 18). Most of the inflammatory changes had disappeared at 10 day PI. The epithelial membrane regained their normal architect and inflammatory reaction disappeared completely. There was no virus isolation or localization at day 10 PI (Table 2).

In the lung, the virus localization was detected in the epithelial cells of secondary or tertiary bronchi and the air capillaries (Fig. 19, 20). There was no obvious histopathological changes observed. The virus isolation was also positive between 8 hour PI and 7 day PI (Table 2, 3).

## DISCUSSION

Immunohistochemnical techniques have gained considerable importance in disease diagnosis and in histopathological studies of the infected tissue. The advent of monoclonal antibodies further adds to the sensitivity and specificity of the reaction.

In the present work virus distribution and histopathologic changes in lungs and tracheas of NDV infected chicken were studied using immunohistochemical techniques. The course of the infection was observed for 10 days. It was possible to conduct the following studies; immunofluorescence detection of NDV in infected tissue; comparative study of monoclonal antibody versus polyclonal antibody; virus isolation versus immuno-histological techniques.

Immunofluorescent study with polyclonal antisera confirmed the previous observations of some workers (Braune, & Gentry, 1965; Heuschele, & Easterday, 1970; Brown, & Cunningham, 1971). However due to unavoidable heterogeneity of commercially available polyclonal antisera, we faced nonspecific staining problems. Practically it was impossible to standardize each and every batch of polyclonal antiserum due to the heterogeneity of antibodies. The polyclonal antiserum obtained from USDA gave better results and it was possible to localize virus in the cytoplasm of

infected epithelial cells of respiratory tract well in advance of the sign of clinically visible illness (Fig 3, 4, 5).

The bright granular fluorescent staining with PAb was a common feature in the infected cells. The use of monoclonal antibody as a highly specific reagent in NDV diagnosis was very successful and promising. A diffuse and speckled fluorescence within the cytoplasm of infected epithelial cells was observed with almost negligible background staining (Fig 6,7). The higher staining intensity and the granular cytoplasmic staining the PAb due to the multiple antibodies directed against multiple epitopes of NDV (Fig 3, 4). Since monoclonal antibodies can recognize a specific epitope of the virus, a diffuse and speckled immunofluorescent with less intensity was observed (Fig. 2, 6).

The selection of a suitable immunoenzymatic technique was a critical issue. For instance streptavidin peroxidase and ABC methods showed considerable nonspecific staining which may be due to chemical modification of the enzyme. An unlabeled antibody method, APAAP was one of the most sensitive technique we applied (Cordrll, et al. 1984) (Fig. 1).

APAAP staining using monoclonal antibody also detected viral antigens in the paraffin-processed lungs and tracheal sections. The sensitivity and specificity of this technique

was similar to immunofluorescent staining (Table 2, 3). The relative number of positive cells per field indicated the duration and extent of infection.

The most interesting aspect of APAAP staining is the fact that the pathological changes can be investigated along with the actual location of antigen in situ. In the tracheal section at 8 hr PI, for instance, viral antigen was frequently localized in the cytoplasm of epithelial cell with partial deciliation (Fig. 9). There was complete deciliation at 36 hr PI with intra and extra cellular localization of antigen (Fig. 11). With epithelial vacuolation observed at 48 hr PI (Fig. 11, 12), the antigen was also present in subepithelial tissue (Fig. 13). Extensive epithelial sloughing occurred at 120 hr PI (Fig. 16,17). This seemed to be a peak of infection which subsides at day-seven (Fig. 18).

The identification of pathogenic strain of virus with the use of their specific monoclonal antibody in situ is a convenient method. It was possible to locate NDV antigen with immunofluorescence technique within two hour and with APAAP technique within same day as compare to the conventional viral identification and isolation method which is cumbersome and time consuming. Since antigenic variant of NDV exist in the field, the use of cocktail of several monoclonal antibodies reacting with all viruses of the group is suggested followed by individual MAb for detection of

pathotype. Application of APAAP method in rutin diagnoses of early outbreaks and epidemiological studies of NDV is suggested.

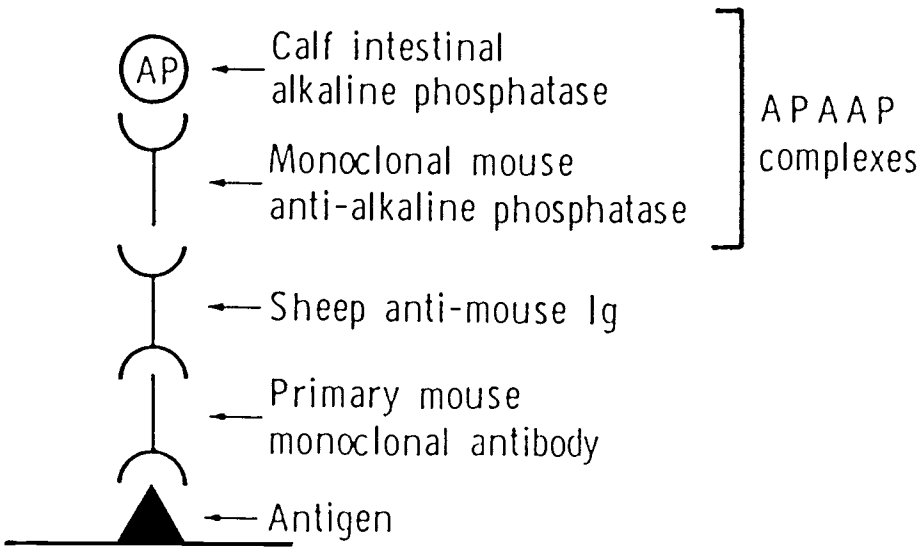


Figure 1. Immune complex of Alkaline phosphatase and monoclonal anti-alkaline phosphates (APAAP).

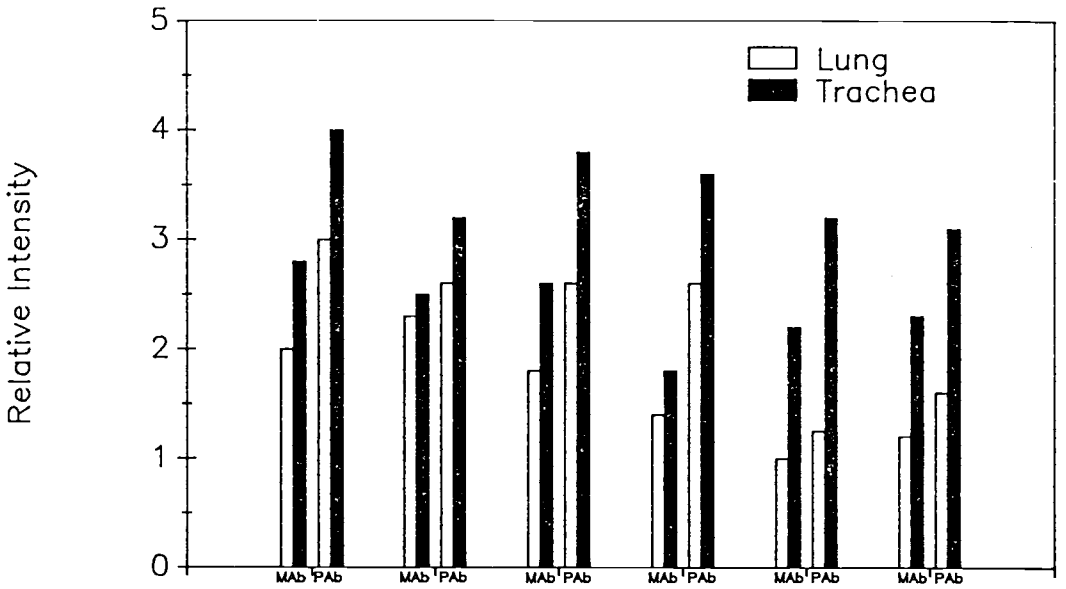


Figure 2. MAb and PAb immunofluorescence staining intensity comparison of lungs and tracheal impression smears.

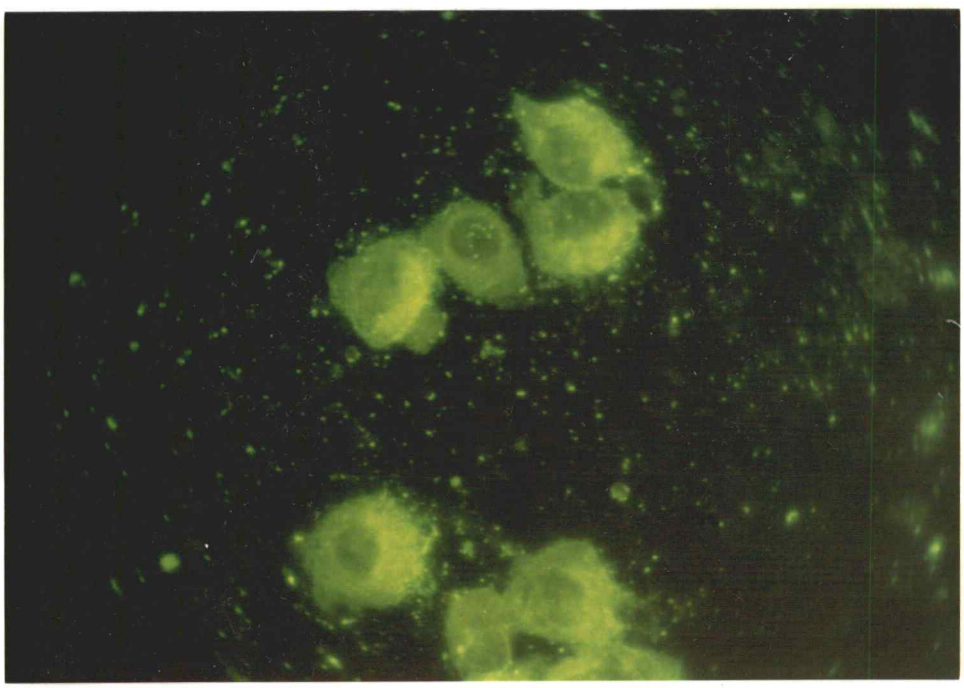

Figure 3. Immunofluorescence staining of tracheal smear using polyclonal antibody. 120 hr PI.

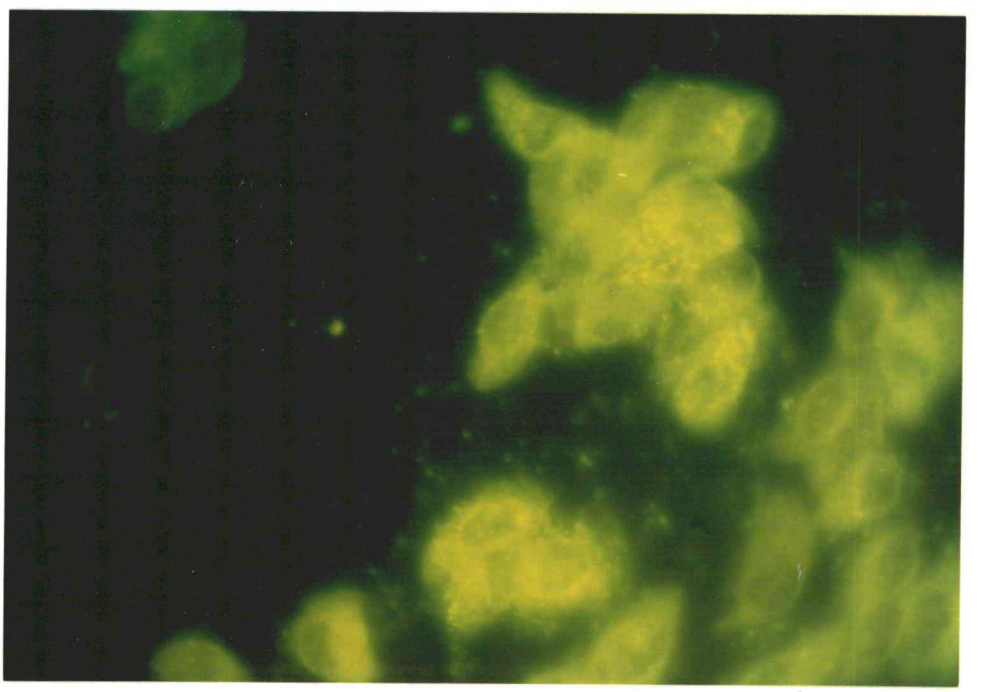

Figure 4. Immunofluorescence staining of tracheal smears with polyclonal antibody. 120 hr PI.

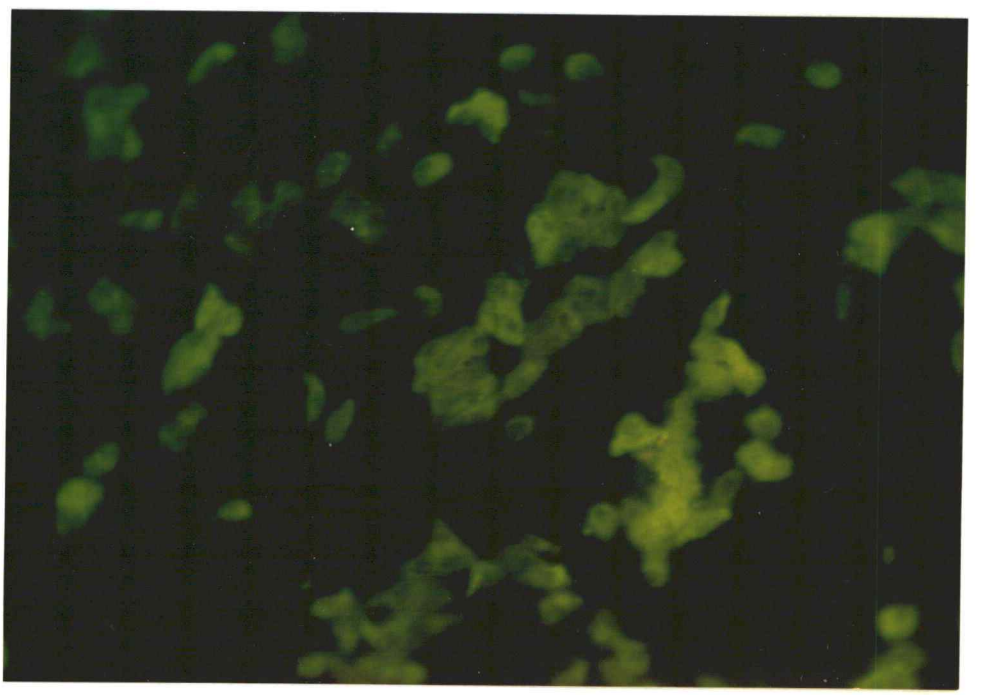

Figure 5. Immunofluorescence staining of lung smears with polyclonal antibody. 120 hr PI.

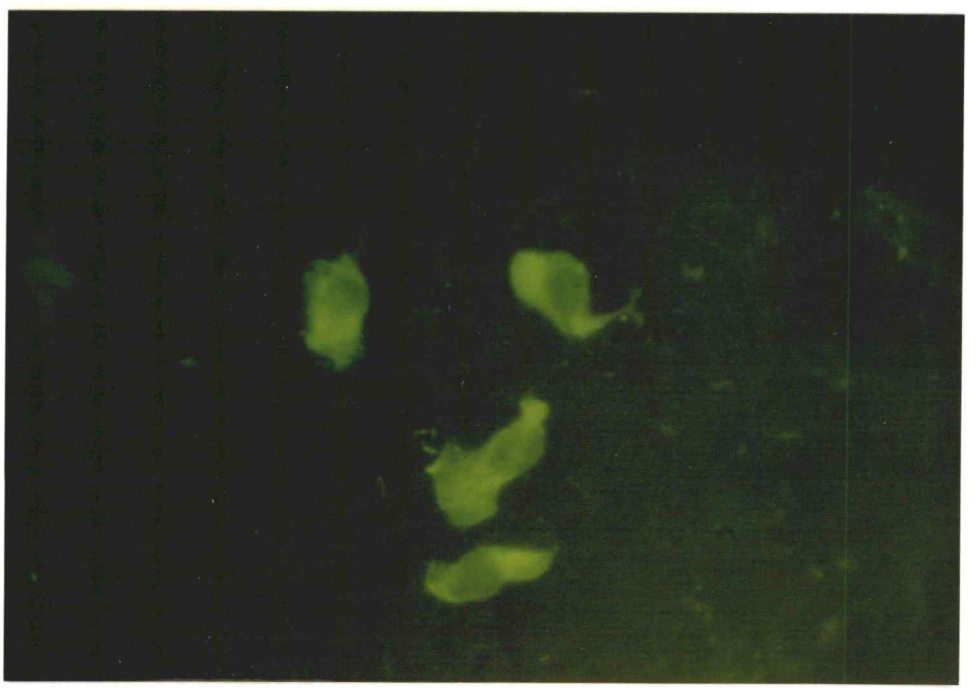

Figure 6. Immunofluorescence staining of tracheal smears with monoclonal antibody. 120 hr PI.

Figure 7. Immunofluorescence staining of lung smears with monoclonal antibody. 120 hr PI.

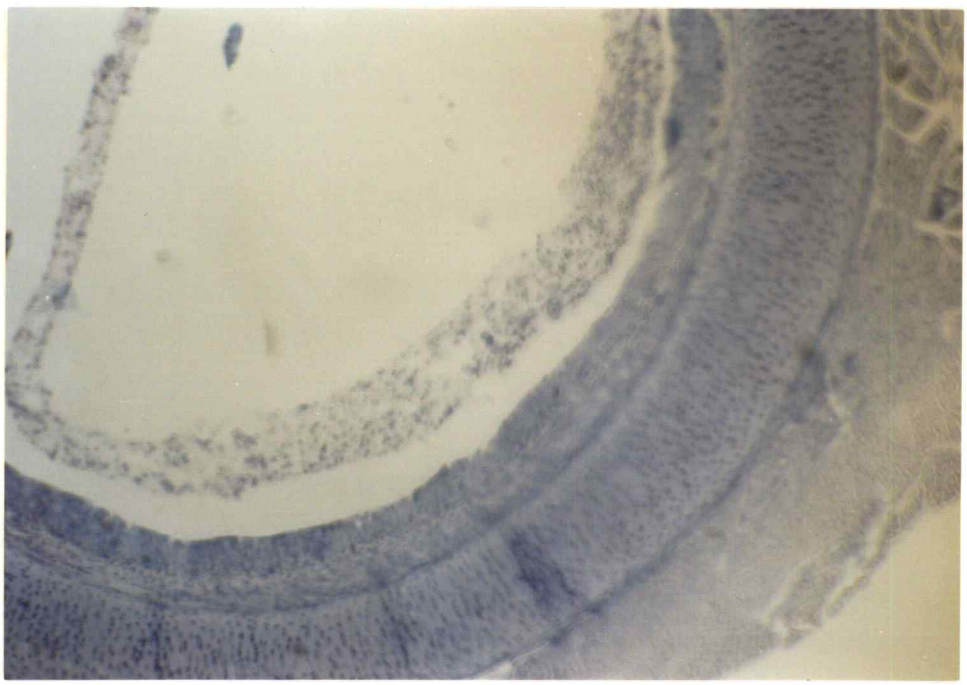

Figure 8. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin embedded section of trachea from control chicken. 24 hr PI.

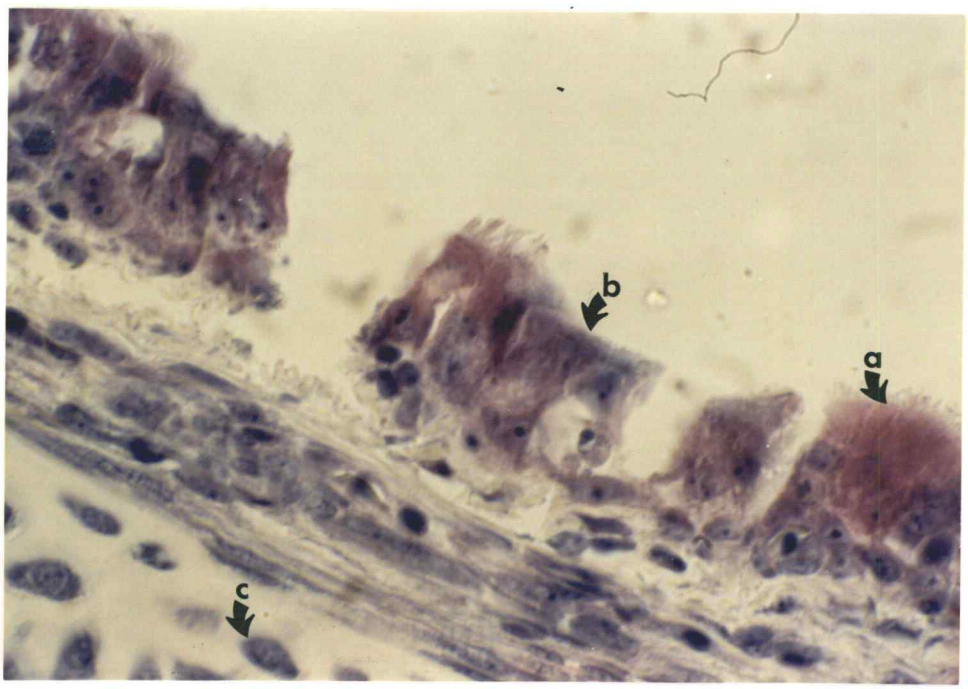


Figure 9. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin section of the trachea obtained from an infected chicken. (a) Red staining in the cytoplasm of tracheal epithelium. (b) Partial deciliation (c) Traheal cartilage counterstained with contrast blue. 8 hr PI.

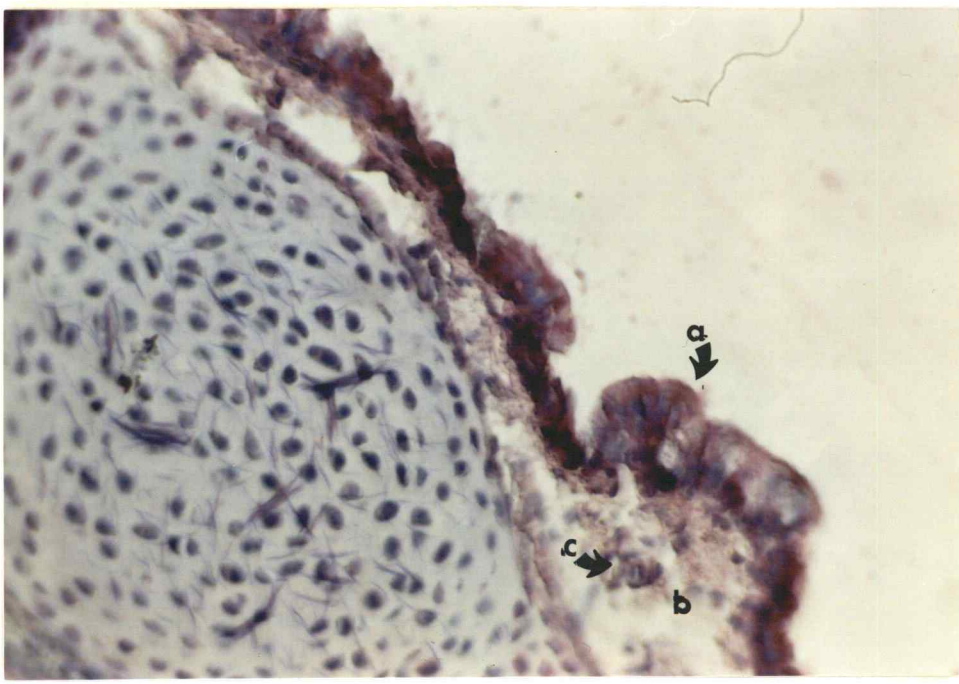


Figure 10. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin section of the trachea obtained from an infected chicken. (a) separation of mucosa (b) submucosal edema (c) cellular infiltration. 24 hr PI.

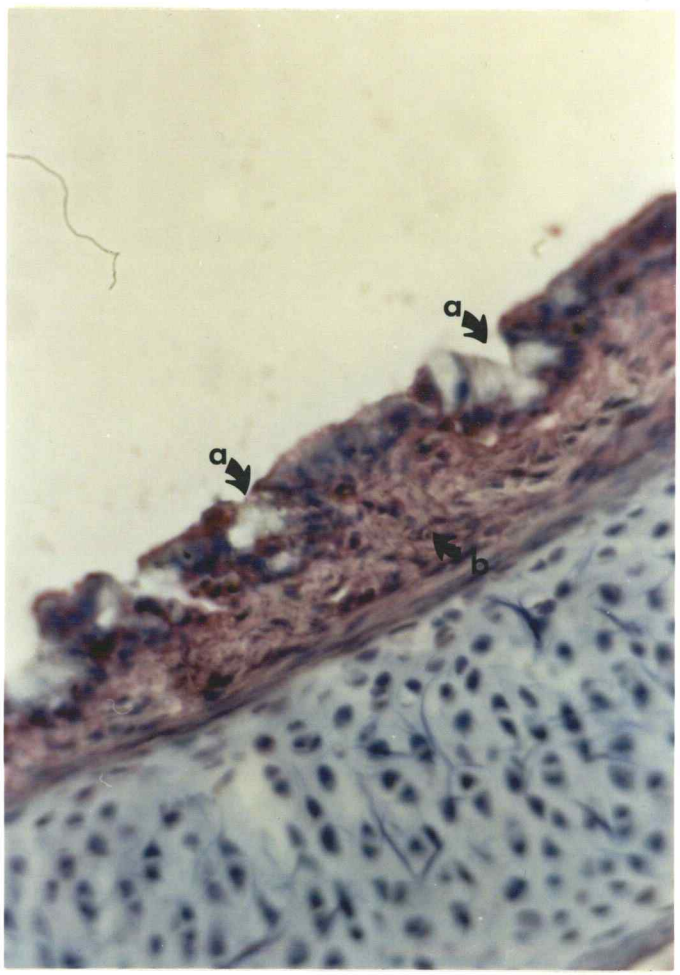


Figure 11. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin section of the trachea obtained from an infected chicken. (a) vacuolation and (b) intra and extra cellular localization of antigen.
48 hr PI.

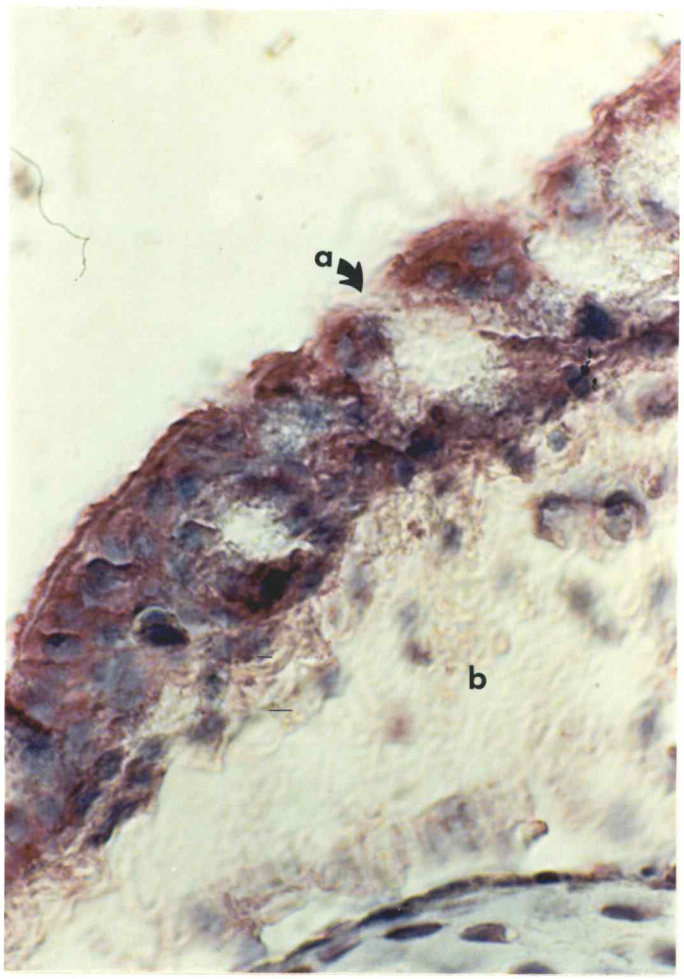


Figure 12. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin section of the trachea obtained from an infected chicken. (a) vacuolation (b) marked edema. 48 hr PI.

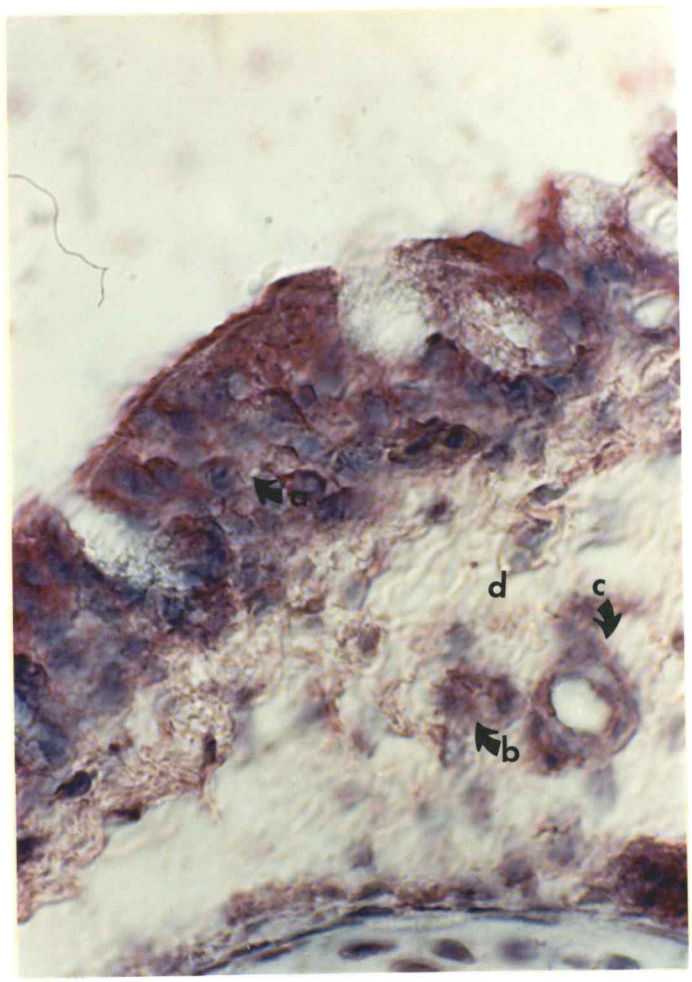


Figure 13. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin section of the trachea obtained from an infected chicken. (b) Inflammatory reaction (c) congestion of blood vessel (d) marked edema.
48 hr PI.

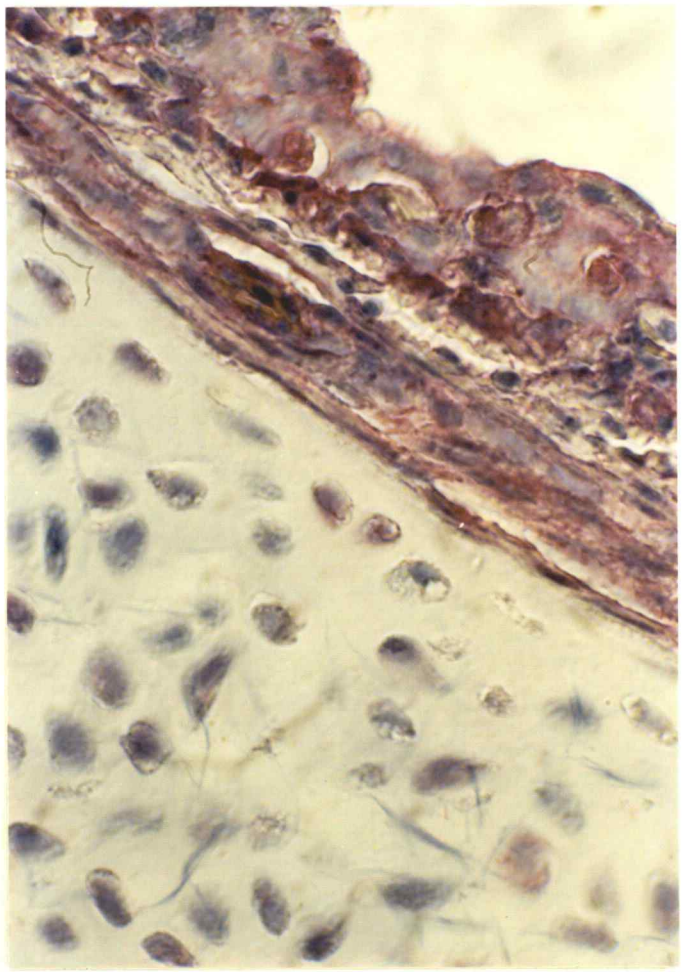

Figure 14. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin embedded section of trachea from infected chicken.
72 hr PI.

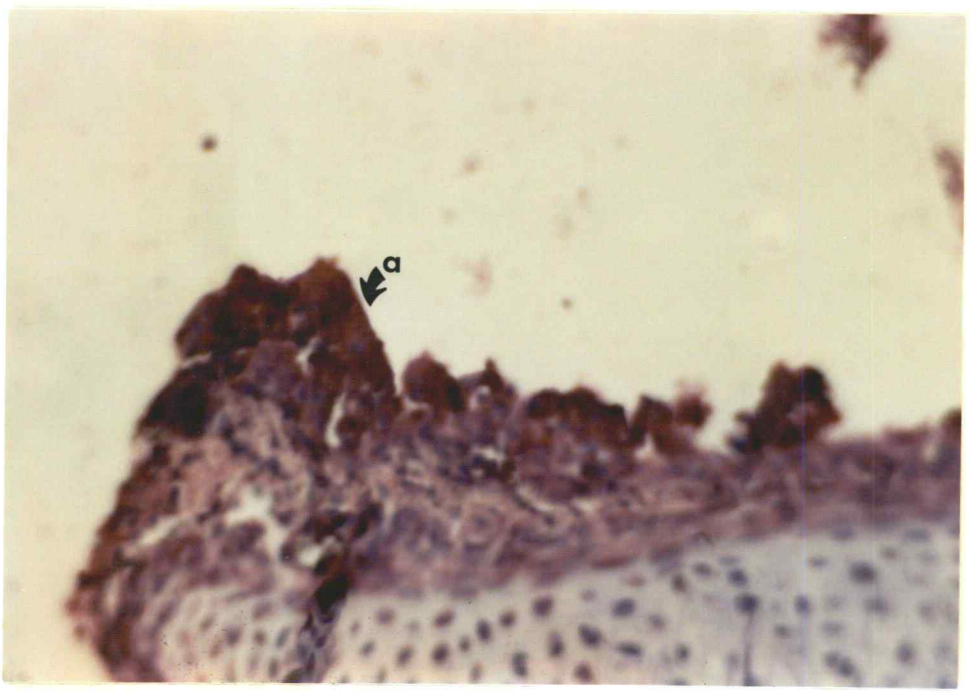


Figure 15. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin section of the trachea obtained from an infected chicken. (a) hyperplasia, necrosis and sloughing of tracheal epithelium.
120 hr PI.

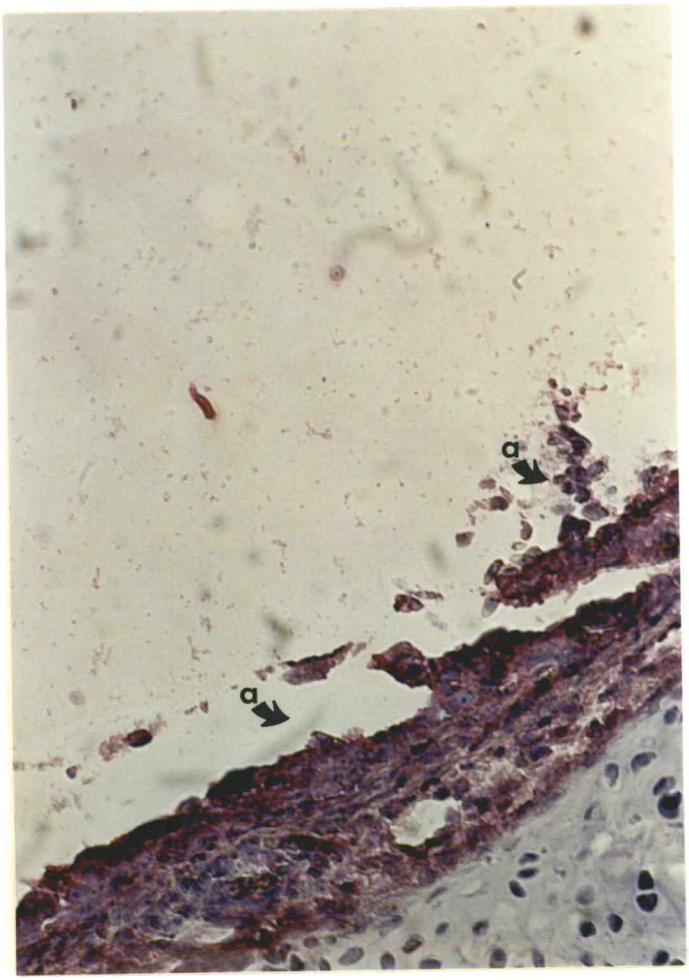


Figure 16. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin section of the trachea obtained from an infected chicken. (a) hyperplasia, necrosis and sloughing of tracheal epithelium.
120 hr PI.

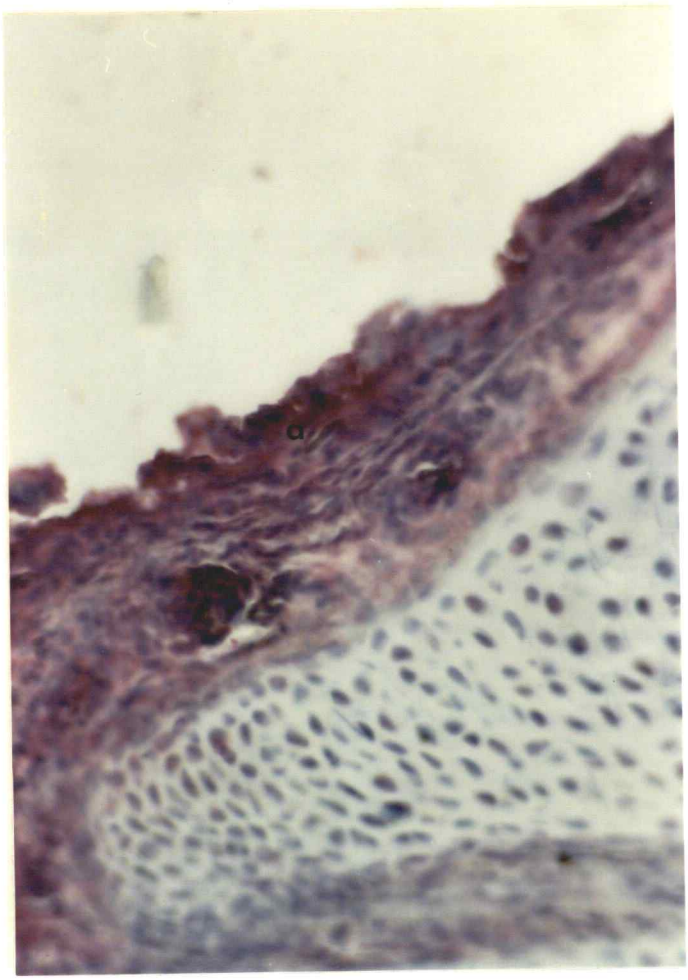


Figure 17. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin section of the trachea obtained from an infected chicken. (a) hyperplasia, necrosis and sloughing of tracheal epithelium.
120 hr PI.

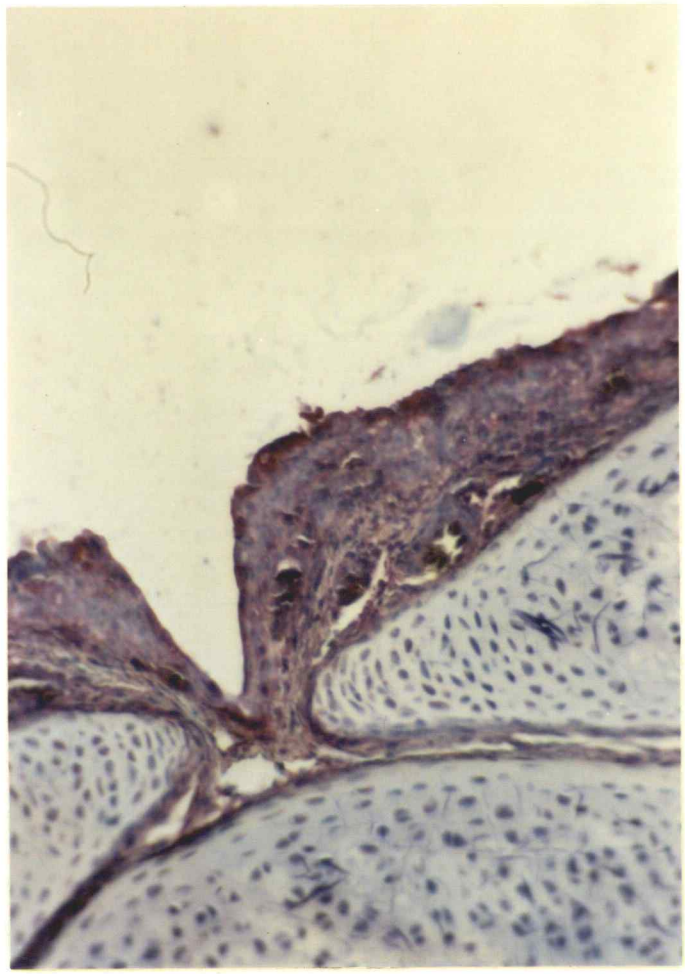

Figure 18. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin section of the trachea obtained from an infected chicken. Red staining of ND antigen with necrosis, cellular infiltration.
7 day PI.

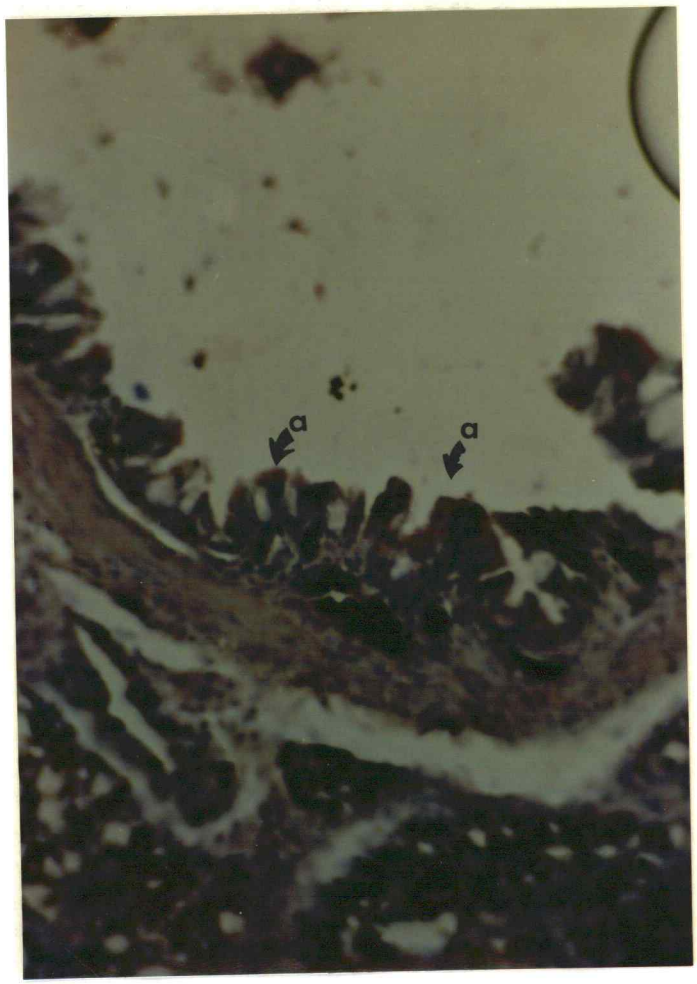


Figure 19. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin embedded section of lung from infected chicken. (a) red staining of epithelial cells of secondary bronchi
120 hr PI.

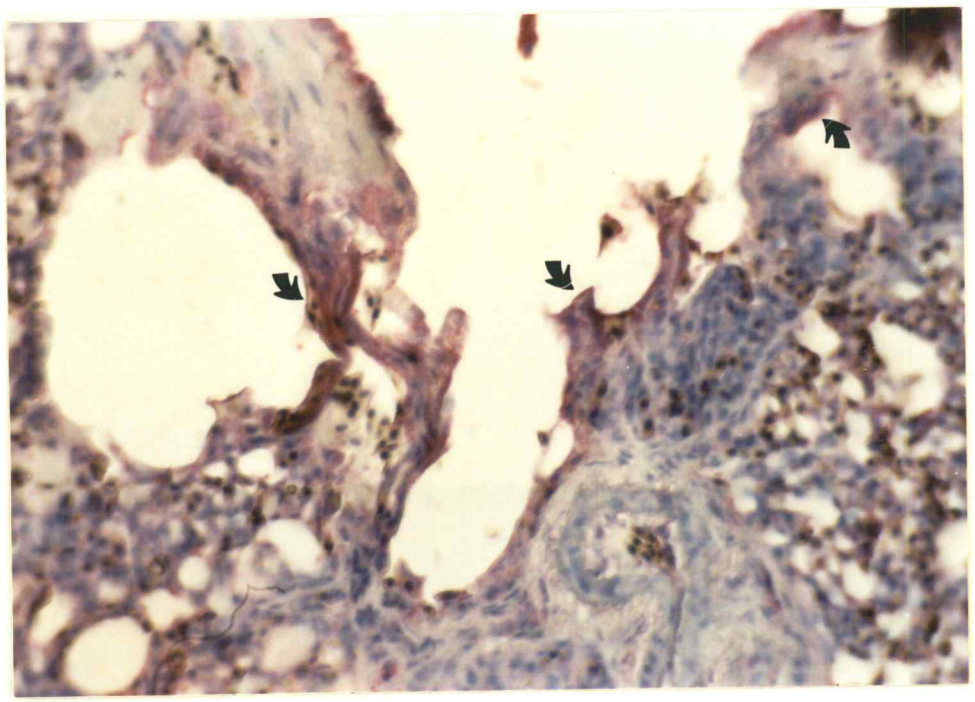

Figure 20. Alkaline phosphatase-anti-alkaline phosphatase staining of paraffin embedded section of lung from infected chicken.
localization of ND antigen in the endothelial cells of air capillary.
120 hr PI.

Table 1. Immunohistochemistry and virus isolation study in one-week-old SPF chicken lung and trachea saved during virus titration.

| GROUP | Dilution[A] | Virus isolation[B] | | IF[C] | | | |
|---|---|---|---|---|---|---|---|
| | | | | MAb[D] | | PAb[E] | |
| | | L | T | L | T | L | T[F] |
| A | $10^{-1}$ | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 |
| B | $10^{-2}$ | 2/4[G] | 4/5 | 3/4 | 5/5 | 3/4 | 5/5 |
| C | $10^{-3}$ | 6/6 | 6/6 | 5/6 | 6/6 | 6/6 | 6/6 |
| D | $10^{-4}$ | 5/6 | 6/6 | 5/6 | 6/6 | 5/6 | 6/6 |
| E | $10^{-5}$ | 2/6 | 2/4 | 4/6 | 3/4 | 4/6 | 3/4 |
| F | $10^{-6}$ | 4/6 | 6/6 | 4/6 | 6/6 | 5/6 | 6/6 |
| G | control | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |

[A] Ten fold stock virus dilution inoculated intra-tracheal.

[B] Positive sample/total

[C] Immuno fluorescent staining was graded from 0 (negative) and 1 to 4 (positive).

[D] Monoclonal antibody B-79.

[E] Polyclonal serum.

[F] Impression smears were made from lung (L) and trachea (T).

[G] Contaminated samples were not counted.

Table 2. Immunohistochemistry and virus isolation study of lung and tracheal samples at indicated time following NDV infection.

| Group | Sampling Time | Virus Isolation[B] | | APAAP[C] | | IF[D] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | MAb[E] | | MAb | | PAb[F] | |
| | | L | T | L | T[G] | L | T[H] | L | T[H] |
| A | 0hr | 0/5 | 1/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| B | 4hr | 1/5 | 2/5 | 1/5 | 1/5 | 1/5 | 1/5 | 1/5 | 1/5 |
| C | 8hr | 4/5 | 4/5 | 2/5 | 5/5 | 2/5 | 5/5 | 2/5 | 5/5 |
| D | 12hr | 5/5 | 5/5 | 2/5 | 5/5 | 2/5 | 5/5 | 3/5 | 5/5 |
| E | 24hr | 5/5 | 4/5 | 2/5 | 5/5 | 2/5 | 5/5 | 4/5 | 5/5 |
| F | 48hr | 4/5 | 5/5 | 2/5 | 5/5 | 2/5 | 5/5 | 3/5 | 5/5 |
| G | 72hr | 4/5 | 5/5 | 3/5 | 5/5 | 3/5 | 5/5 | 3/5 | 5/5 |
| H | 96hr | 5/5 | 4/5 | 3/5 | 5/5 | 3/5 | 4/5 | 4/5 | 5/5 |
| I | 120hr | 4/5 | 4/5 | 2/5 | 5/5 | 4/5 | 5/5 | 4/5 | 5/5 |
| J | 7d | 0/5 | 1/5 | 0/5 | 2/5 | 0/5 | 1/5 | 1/5 | 2/5 |
| H | 10d | 0/5 | 1/5 | 0/5 | 1/5 | 0/5 | 0/5 | 1/5 | 1/5 |
| Control | 0hr | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Control | 24hr | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |

[A] Samples collected at various time interval after intratracheal inoculation of 1000 ID/50 La Sota stock virus.

[B] Positive sample/total.

[C] Alkaline Phosphatase and monoclonal Anti-alkaline phosphatase complex. Staining was graded from 0 (negative) and +1 to 4+ (positive).

[D] Fluorescent staining was graded from 0 (negative) and +1 to 4+ (positive).

[E] Monoclonal antibody B-79 against PMV-1.

[F] Polyclonal serum obtained from chicken immunized by LaSota virus.

[G] Paraffin imbeded sections of lung (L) and trachea (T).

[H] Impression smears of lung (L) and trachea (T).

Table 3. Immunohistochemistry of lung and tracheal sample at indicated time following NDV infection.

| Group | Sampling[A] Time | APAAP[C] MAb[E] L | APAAP[C] MAb[E] T[G] | IF[D] MAb L | IF[D] MAb T | IF[D] PAb[F] L | IF[D] PAb[F] T |
|---|---|---|---|---|---|---|---|
| A | 0hr | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4[B] |
| B | 4hr | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | 1/4 |
| C | 8hr | 2/4 | 4/4 | 2/4 | 4/4 | 3/4 | 4/4 |
| D | 12hr | 3/4 | 4/4 | 3/4 | 4/4 | 3/4 | 4/4 |
| E | 24hr | 3/4 | 4/4 | 3/4 | 4/4 | 3/4 | 4/4 |
| F | 48hr | 3/4 | 4/4 | 3/4 | 4/4 | 3/4 | 4/4 |
| G | 72hr | 3/4 | 4/4 | 3/4 | 4/4 | 3/4 | 4/4 |
| H | 96hr | 3/4 | 4/4 | 3/4 | 4/4 | 4/4 | 4/4 |
| I | 120hr | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| J | 7d | 2/4 | 1/4 | 2/4 | 2/4 | 2/4 | 2/4 |
| Control | 24hr | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |

[A] Samples collected at various time interval after intratracheal innoculation of 1000 ID/50, .1ml La Sota stock virus.

[B] Positive sample/total.

[C] Alkaline Phosphatase and monoclonal Anti-alkaline phosphatase complex. Staining was graded from 0 (negative) and +1 to 4+ (positive).

[D] Immunofluorescence staining of impression smears.

[E] Monoclonal antibody B-79 against PMV-1.

[F] Polyclonal serum obtained from chicken immunized by LaSota virus.

[G] Paraffin imbeded sections of lung and trachea.

**BIBLIOGRAPHY**

Alexander DJ. Avian paramyxoviruses. Vet Bull 50:737-752. 1980.

Alexander DJ. Avian paramyxoviruses other than Newcastle disease virus. World's poult Sci J 38:97-104. 1982.

Alexander DJ, Collins MS. Arch. Virol. 67:309-323. 1981.

Alexander DJ, Hinshaw VS, Collins MS, Yamane N. Characterization of virus which represent further distinct serotypes (PMV-8 and PMV-9) of avian paramyxoviruses. Arch Virol 78:29-36. 1983.

Alexander DJ. The classification, host range and distribution of avian paramyxoviruses. In: Acute virus infections of poultry. Edited by McFerran JB, Mcnulty MS. Brussels- Luxembourg. 52-66. 1986.

Alexader DJ. Newcastle disease diagnosis. In Newcastle disease. Edited by Alexander DJ. Kluwer Academic publishers London. 147-160. 1988.

Alexander DJ, Manvall RJ, Kemp PA, Parsons G, Collins MS, Brockman S, Russell PH, Lister SA. Use of monoclonal antibody in the characterization of avian paramyxovirus type 1 (Newcastle disease virus) isolates submitted to an international reference laboratory. Avian Pathol 16:553-565. 1987.

Alexander DJ, Russell PH, Clooins MS. Paramyxovirus type 1 infections of racing pigeons. 1. Characterization of isolated viruses. Vet Rec 114:444-446. 1984.

Allan WH, Lancaster JE, Toth B. In: Methods of the examination of poultry Biologics. National Academy of Science Washington D.C. 1973.

Asdell MK, Hanson RP. Newcastle disease transmission mechanism of the chicken. Am J Vet Res 21:128-132. 1960.

Banerjee AK. The transcription complex of vesicular stomatitis virus. Cell 48:363-364. 1987.

Bang FB. Studies on Newcastle disease virus. 1. Characters of the virus it self with particular reference to electron microscopy. J Exp Med 88:251-266. 1948.

Bang FB, Foard M, Bang BG. Acute Newcastle viral infection

of the upper respiratory tract of the chicken: A modle for the study of environmental factors on upper respiratory tract infection. Am J Path 76:333-348. 1974.

Beard CW, Hanson RP. In: Disease of poultry 8th ed. Edited by Hofsted MS, Baners HJ, Clanek BW, Reid WM, Yoder HW, Jr. Iowa State Univ. Press Ames. 452-470. 1984.

Braune MO, Gentry RF. Standardization of fluorescent antibody technique for the detection of avian respiratory viruses. Avian Dis 9(4):535-545. 1965.

Brown JL, Cunningham CH. Immunofluorescence of avian Infectious bronchitis virus and Newcastle disease virus in singly and dually infected cell culture. Avian Dis 15(4):923-935. 1971.

Cavangh D. Monoclonal antibody and nucleic acid probes for diagnosis of avian disease. Avian Dis 30:12-19. 1985.

Chambers P, Millar NS, Bingham RW, Emmerson PT. Molecular cloning of complementary DNA to Newcastle disease virus, and nucleotide sequence analysis of the junction between the genes encoding the hemagglutinin-neuraminidase and the large protein. J Gen Virol 67:475-486. 1986.

Cho BR, Snyder DB, Lana DP, Marquardt WW. An immunoperoxidase monoclonal antibody staining for rapid diagnosis of infectious bursal disease. Avian Dis 31:538-545. 1987.

Choppin PW, Compans RW. In: Comprehensive virology. Edited by Freenkel-Conrat H, and Wagner RR. Plenum New York Vol.4:95-178. 1975.

Collins PL, Hightower LE, Ball LA. Transcriptional map for Newcastle disease virus. J Virol 35:682-693. 1980.

Colonno RJ, Stone HO. Isolation of transcriptive complex from Newcastle disease virus. J Virol 19:1080-1089. 1976.

Coons AH, Leduc EH, Connolly JM. Studies on antibody production. 1. A method for histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J Exp Med. 102:49-59. 1955.

Doyl TM. A hitherto unrecorded disease of fowl due to filtrable virus. J Comp Path 40:144-269. 1927.

Erdei J, Erdei J, Bachir K, Kaleta EF, Shortridge KF, Lomniczi EF. Newcastle disease vaccine (LaSota) strain specific monoclonal antibody. Arch Virol 96:265-269. 1987.

Feller U, Dougherty RM, DeStefano HS. Morphogenesis of Newcastle disease virus in chorioallantoic membrane. J Virol 4:753-762. 1969.

Finch JT. Observation of structure of the nucleocapsid of some paramyxoviruses. J Gen Virol 141-150. 1970.

Hamaguchi M, Yoshida T, Nishikawa K, Naruse H, Nagai Y. Transcriptive complex o newcastle disease virus. 1. Both L and P proteins are required to constitute an active complex. Virology 128:105-117. 1983.

Hanson RP. In: Isolation and identification of avian pathogens. Edited by Hitchner SB. Am. Assoc. Avian Path. College station texas. 1980.

Heuschele WP, Easterday BC. Local immunity and persistence of virus in the tracheas of chickens following infection with Newcastle disease virus. II. Immunofluorescent and histopathological studies. The J of Inf Dis 121(5):497-504. 1970.

Iorio RM, Bratt MA. Monoclonal antibodies to Newcastle disease virus: Delineation of four epitopes on the HN glycoprotein. Journal of Virology. 48:440-450. 1983.

Iorio RM, Borgman JB, GLickman BL, Bratt MA. J Gen Virol 67:1393-1403. 1986.

Kaleta EF, Siegmann O, Jank-Ladwig, Glunder, G. Isolation and biological properties of virulent subpopulations from Lentogenic Newcastle disease virus strains. Comp Immunol Microbiol Inft Dis 2:485-496. 1979.

Katho H. Pathological studies on Newcastle disease: Laryngotracheal and conjunctival lesions caused by so-called asian type Newcastle disease virus. Jap J Vet Sci. 39:15-26. 1977.

Kessler N, Aymard M, Calvet A. Study of a new strain of Paramyxoviruses isolated from wild ducks: antigenic and biological properties. J Gen Virol 43:273-282. 1979.

Kingsbury DW, Darlington RW. Isolation and properties of Newcastle disease virus nucleocapsid. J Virol 2:248-255. 1968.

Kingsbury DW. Replication and function of myxovirus ribonucleic acids. Progr Med Virol 12:49-77. 1970.

Klenk HD, Choppin PW. Plasma membrane lipids and

parainfluenza virus assembly. Virology 40:939-947. 1970.

Klenk HD, Compans RW, Choppin PW. An electron microscopic study of the presence or absence of neuraminic acid in enveloped viruses. Virology 42:1158-1162. 1970.

Kotani T, Odagiri Y, Nakamura J, Horiuchi T. Pathological changes of tracheal mucosa in chickens infected with Lentogenic Newcastle disease virus. Avian Dis 31:491-497. 1987.

Kraneveld FC. A poultry disease in Dutch east india. Ned Indish B1 Diergeneeskd 38:448-451 1926.

Kurilla MG, Stone HO, Keene JD. RNA sequence and transcriptional properties of 3' end of Newcastle disease virus genom. Virology 145:203-212. 1985.

Lana DP, Snyder DB, King DJ, Marquardt WW. Characterization of a battery of monoclonal antibodies for differentiation of Newcastle disease virus and paramyxovirus-1 strains. Avian Dis 32:273-281. 1988.

Lancaster JE. In: Newcastle Disease. Monograph No. 3. Canada Dept of Agriculture, Ottawa. 1966.

Lancaster JE. Newcastle disease: Pathogenesis and diagnosis. Words poultry science 16:26-33. 1981.

Levine S, Sagik BP. The interactions of Newcastle disease virus (NDV) with chick embryo culture cells. Virology 2:57-68. 1956.

Mason DY, Sammons RE. Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. J Clin Pathol 31:454-460. 1978.

Mason DY. Immunohistochemical labeling of monoclonal antibodies by APAAP immunoalkaline phosphatase technique. In: Techniques in immunocytochemistry. Edited by Bullock GR. Academic press. vol 3:25-42. 1985.

Matsumoto T. Assembly of paramyxoviruses. Microbiol Immunol 26:285-320. 1982.

Meiseiman N, Kohn A, Danon DJ. Cell science 2:71-76. 1967.

Meulemans G, Gonze M, Carlier MC, Petit P, Burny A, Long Le. Evaluation of the use of monoclonal antibodies to hemagglutinatinin and fusion glycoprotein of Newcastle disease virus for virus identification and strain differentiation purpose. Arch Virol 92:55-62. 1987.

Nagai Y, Klenk HD, RoTT R. Proteolytic cleavage of viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology 72:494-508. 1976.

Nagai Y, Ogura H, Klenk HD. Studies on the assembly of the envelop of Newcastle disease virus. Virology 69:523-538. 1976.

Nagai Y, Yoshida T, Yoshii S, Maeno K, Matsumoto T. Modification of normal cell surface by smooth membrane preparations from BHK-21 cells infected with Newcastle disease virus. Med Microbiol Immunol 161:175-1988. 1975.

Nishikawa K, Morishima T, Yoshida T, Hamaguchi M, Toyoda T, Nagao Y. Antigenic characterization of internal proteins of Newcastle disease virus by monoclonal antibodies. Virus research 7:83-92. 1987.

Orsborn JS. Newcastle disease. In: Foregn animal disease symposium, Cornell University, Ithaca, New York. 1977.

Oxford J. The use of monoclonal antibodies in virology. J Hygiene 88:361-388. 1982.

Portner A, Murti KG. Localization of P NP and M proteins on sendai virus nucleocapsid using immuno gold labelling. Virology 150:469-478. 1986.

Rott R. In: Newcastle Disease Virus. An evolving pathogen. Edited by Hanson RP. 133-46 Univ. Wisconsin press, Madison. 133-146. 1964.

Rott R, Schafer W. Fine structure of subunits isolated from Newcastle Disease virus (NDV). Virology 14:298-299. 1961.

Russell PH, Alexander DJ. Antigenic variation of Newcastle disease virus strains detected by monoclonal antibodies. Arch Virol 75:243-253. 1983.

Sadasiv EC, Piela TH, Chang PW. Detection of Latent Avian Adenovirus-Associated virus proteins in chicken cell. Avian Dis 33:125-133. 1989.

Scheid A, Choppin PW. Two disulfide linked polypeptide chains constitute the active F protein of Paramyxoviruses. Virology 80:54-66. 1977.

Scheid A, Choppin PW. Isolation and purification of the envelop proteins of Newcastle disease virus. J Virol 11:263-271. 1973.

Singh KV, El-Zein A. Viral proliferation patterns of a velogenic (VLT), a Mesogenic (Komarove), and a Lentogenic (F) strain of newcastle disease virus. Poultry Sci 57:1563-1566. 1978.

Srinivasappa GB, Snyder DB, Marquardt WW, King DJ. Isolation of a monoclonal antibody with specificity for commonly employed vaccine strains of Newcastle disease virus. Avian Dis 30:562-567. 1987.

Sumner BEH. In: Basic histochemistry. John Wiley & Sons. New York 1988.

Weller TH, Coons AH. Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro. Proc Soc Exp Biol Med 86:789-794. 1954.

Wheelock EF. Intracellular site of Newcastle disease virus nucleic acid synthesis. Proc Soc Exp Biol Med 114:56-60. 1963.